FEBS 29518 FEBS Letters 579 (2005) 2727–2730

Scallop DMT functions as a Ca^{2+} transporter

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Received 21 January 2005; revised 10 March 2005; accepted 10 March 2005

Available online 9 April 2005

Edited by Stuart Ferguson

Abstract **We identified a DMT (divalent metal transporter) homologous protein that functions as a Ca^{2+} transporter. Scallop DMT cDNA encodes a 539-amino-acid protein with 12 putative membrane-spanning domains and has a consensus transport motif in the fourth extracellular loop. Since its mRNA is significantly expressed in the gill and intestine, it is assumed that scallop DMT transports Ca^{2+} from seawater by the gill and from food by the intestine. Scallop DMT lacks the iron-responsive element commonly found in iron-regulatory proteins, suggesting that it is free of the post-transcriptional regulation from intracellular Fe^{2+} concentration. Scallop DMT distinctly functions as a Ca^{2+} transporter unlike other DMTs, however, it also transports Fe^{2+} and Cd^{2+} similar to them.**

Keywords: Ca^{2+} transporter; Cd^{2+} accumulation; Divalent cation transporter; Scallop

1. Introduction

Metal ions are essential for a variety of biological systems in animals. Metal ions are transported into cells by membrane associated proteins including divalent metal transporters (DMTs), which have been identified from various species [1]. DMT1 (also called DCT1 or Nramp2) was reported as a homologous protein of Nramp which was implicated in natural resistance to infection by intracellular parasites in mouse [2–4]. DMT1 was identified in rat as DCT1 through expression cloning to search for an mRNA that promoted Fe^{2+} uptake activity [5]. Mutations in the DMT1 gene caused a defect in intestinal iron absorption and red cell iron utilization in rat and mouse, suggesting that DMT1 plays an important role in iron absorption by intestinal cells [6–8]. The presence of an iron-regulatory element (IRE) in mammalian DMTs suggests that DMT mRNA is implicated in post-transcriptional regulation by intracellular Fe^{2+} concentration [9,10]. Accordingly, iron deficiency in rat induced a remarkable accumulation in mRNA level of DMT1 due to the function of IRE [5,11].

Some marine organisms accumulate metal ions in extraordinarily high concentration. Some ascidian species accumulates vanadium in concentration up to 10^7-fold that of seawater in specific blood cells called vanadocytes [12]. Scallop accumulates cadmium at the concentration of more than 10 μg/g wet tissue in the hepatopancreas that corresponds to 10^7-fold in concentrations up to that of seawater [13,14]. Since DMT1 transports a variety of divalent metal ions including Cd^{2+} [5,15,16], we tried to isolate a gene encoding a scallop DMT (scDMT, DMT1 homologous protein in scallop) possibly involved in the cadmium accumulation. As a result, we successfully identified scDMT gene and characterized the metal ion transport activity of scDMT. Interestingly, our results suggest that scDMT has an ability to transport Ca^{2+} different from other DMTs so far reported, while it transported Fe^{2+} and Cd^{2+} similar to other DMTs.

2. Materials and methods

2.1. Materials

Scallops, *Mizuhopecten yessoensis*, cultured in Hokkaido in Japan (10–12 cm) were obtained alive. Gill, mantle, adductor muscle, hepatopancreas, gonad (testis) and kidney were collected, immediately frozen in liquid nitrogen and stored at −80 °C. All other materials used in this study were of the highest quality commercially available.

2.2. cDNA cloning

Routine molecular cloning techniques were carried out according to the standard procedures [17]. PCR was performed using a single-stranded cDNA generated from scallop hepatopancreas total RNA extracted with guanidine isothiocyanate/cesium chloride method. One microgram of total RNA was transcribed into cDNA with a SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech) according to the manufacturer's protocol. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with degenerate primers based on the conserved amino acid sequences of human DMT (Nramp2/DMT1/DCT1, DDBJ Accession No. AB004857) [18] and *Caenorhabditis elegans* DMT (Smf2, DDBJ Accession No. U23525) with Advantage 2 polymerase mix (BD Biosciences Clontech). Nested PCR was carried out using the resultant PCR products. Oligonucleotides used for primers in PCR cloning were: 5′-GAYATGCARGARGTNATHGG-3′ (sense) and 5′-ACRAANCCYTCCATNACRAAYTG-3′ (antisense) for RT-PCR, and 5′-GAYATGCARGARGTNATHGG-3′ (sense) and 5′-CCNGWRTANGTNCCNGTCAT-3′ (antisense) for following nested PCR. Both PCRs were performed for 30 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min) after a hot start for 1 min at 94 °C, with a Program Temp Control System PC-700 (Astec). Amplified DNAs were subcloned into pGEM-T Easy Vector (Promega) and

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Abbreviations: CTM, consensus transport motif; DCT, divalent cation transporter; DMT, divalent metal transporter; IRE, iron-regulatory element; Nramp, natural resistance associated macrophage protein; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; scDMT, scallop DMT

0014-5793/$30.00 © 2005 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2005.03.082

sequenced. cDNA encoding scDMT was obtained by the rapid amplification of cDNA ends (RACE) technique using the SMART™ RACE cDNA Amplification Kit. Nucleotide sequences were determined with a Dye Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA, USA).

2.3. RT-PCR analysis

Total RNA was extracted from the gill, mantle, adductor muscle, hepatopancreas, gonad (testis), kidney and intestine using Sepasol-RNA I (Nacalai tesque) according to the manufacturer's protocol, and treated with DNase (Promega) to remove contaminating genomic DNA. Analysis of scDMT mRNA expression in scallop tissues was performed by RT-PCR with Blend Taq (Toyobo) and scDMT-specific primers (sense, 5′-CGATGGATCCACCATGACGACATC-GGTGAA-3; antisense, 5′-GCTTCAAGTTTCCTCAGCCCGTAA-CGGTCC-3′) for 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. The resulting PCR product was visualized by ethidium bromide staining after separation on 1% agarose gels.

2.4. Preparation of RNA-injected oocytes

The 5′ and 3′ untranslated regions of *Xenopus* β-globin gene were inserted upstream and downstream, respectively, of the coding region of scDMT. The expression construct was linearized with *Eco*RV. cRNA was synthesized in vitro by SP6 RNA polymerase using mMESSAGE mMACHINE (Ambion). Small pieces of ovarian lobes were dissected out from cold-anesthetized *Xenopus laevis* and shaken gently at 23 °C for 90 min in a solution (88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 7.5 mM Tris–HCl, pH 7.6) containing 1 mg/ml collagenase (Wako Pure Chemicals). Healthy-looking oocytes greater than 1 mm in diameter were selected and injected with 25 ng/cell of scDMT mRNA. The injected oocytes were incubated at 23 °C for 5 days in sterile modified Barth's saline (88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 7.5 mM Tris–HCl, pH 7.6) supplemented with 10 U/ml penicillin and 10 μg/ml streptomycin.

2.5. Electrophysiological recordings

The Ca^{2+}-containing medium is usually used for the electrophysiological assay of *Xenopus* oocytes. But we measured the activity in the Ca^{2+}-free medium as well to avoid the effect of Ca^{2+}, since Ca^{2+} was reported to suppress the activity of rat DMT (DCT1) [5]. In voltage-clamp recording of whole-cell current, oocytes were perfused with the Ca^{2+}-free medium (96 mM NaCl, 2 mM MES, 1.6 mM $MgCl_2$, 100 μM ascorbic acid, 2.5 mM HEPES, and 2.5 mM MES, buffered to pH 5.5 or 7.5 with Tris-base) or the Ca^{2+}-containing medium (96 mM NaCl, 2 mM KCl, 0.6 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM ascorbic acid, 2.5 mM HEPES, and 2.5 mM MES, buffered to pH 5.5 or 7.5 with Tris-base), clamped at −50 mV by two intracellular glass electrodes filled with 3 M KCl and connected to a voltage-clamp amplifier (OC-725C, Warner). These microelectrodes had resistance of 1–2 MΩ. H^+ response was evoked by switching the extracellular perfusion medium from pH 7.5 to pH 5.5. Divalent metal ion response was elicited by a perfusion with the Ca^{2+}-free medium (pH 5.5) or the Ca^{2+}-containing medium (pH 5.5) containing each divalent metal ion.

3. Results and discussion

3.1. cDNA cloning and mRNA expression analysis

Homology search of nucleotide sequences of the cloned cDNA consisting of 2051 bp by use of the BLAST program (DDBJ) indicated that the cDNA might encode scDMT, a DMT1 homologous protein in scallop. The nucleotide sequence of scDMT has been deposited to DDBJ/EMBL/GenBank Nucleotide Sequence Database under Accession No. AB180910. scDMT cDNA encodes a 539-amino-acid protein. The predicted amino acid sequence of scDMT cDNA shows the highest homology of 64.5% with *Takifugu rubripes* solute carrier family 11 protein (DDBJ Accession No. AJ496549) by FASTA. Fig. 1 shows the deduced amino acid sequence of scDMT in comparison with rat and human DMTs by multiple alignments. By use of CLUSTAL W, identities with rat and human DMTs are found to be 58% and 59%, respectively.

The predicted amino acid sequence of scDMT includes 12 putative transmembrane domains, similar to rat and human DMTs, as demonstrated by MENSAT (http://saier-144-37.ucsd.edu/memsat.html) analysis. A putative glycosylation site is demonstrated in the fourth extracellular loop. A consensus

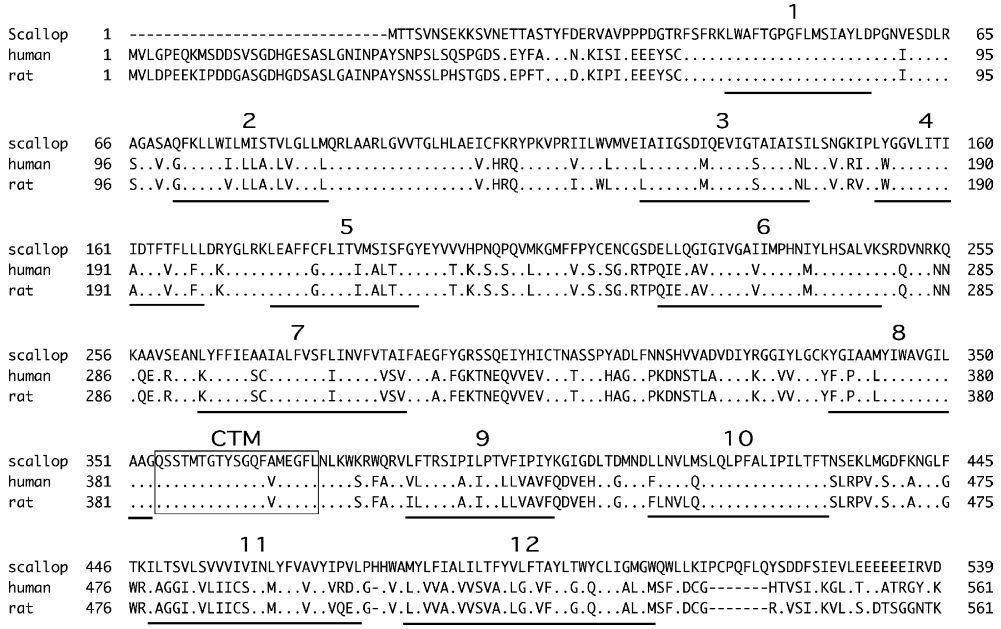

Fig. 1. Deduced amino acid sequence alignment of scDMT and DMT-related transporters. The amino acid sequence of scDMT was aligned with rat and human DMTs (DDBJ Accession Nos. AF008439 and AB004857) by using CLUSTAL W. Dots indicate amino acids identical with those of scDMT. Hyphens indicate gaps. Underlines indicate transmembrane domains predicted. The box indicates a CTM (consensus transport motif).

transport motif (CTM) is demonstrated in the fourth intracellular loop similar to other DMT homologous proteins so far reported [3,5,19].

One of two splice forms of mammalian DMT has an IRE in the 3′ untranslated region similar to transferrin receptor, suggesting that this DMT is regulated after transcription by intracellular Fe^{2+} concentration [5,9]. On the other hand, some DMTs without IREs have been demonstrated to be free of the post-transcriptional regulation from intracellular Fe^{2+} concentration [9,20]. This suggests that scDMT mRNA, which lacks IRE, is not regulated post-transcriptionally by intracellular Fe^{2+} concentration.

3.2. Expression of scDMT in scallop tissues

We examined the expression of scDMT mRNA in various scallop tissues by RT-PCR analysis using scDMT-specific primers. A major band of approximately 760 bp expected to be amplified was detected in the gill and intestine (Fig. 2), suggesting that scDMT might function in transporting divalent metal ions from seawater in the gill and from food in the intestine. Significant expression was also detected in the adductor muscle. This possibly suggests the importance of Ca^{2+} for the contraction of the adductor muscle.

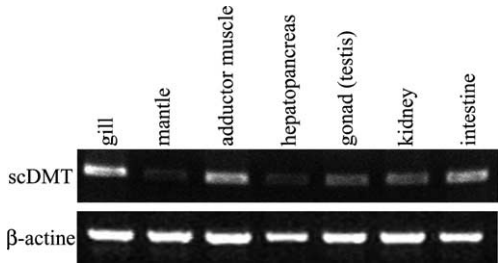

Fig. 2. RT-PCR analysis of scDMT mRNA expression in various scallop tissues. Total RNA was isolated from the gill, mantle, adductor muscle, hepatopancreas, gonad (testis), kidney and intestine, and used in RT-PCR analysis with primers specific for scDMT as described in the text.

3.3. Functional analysis of scDMT in Xenopus oocytes

First, we measured the metal transport activity of scDMT in the Ca^{2+}-free medium. In a water-injected control oocyte, a slight inward shift was observed in the baseline current when extracellular pH was changed from 7.5 to 5.5 (Fig. 3a). However, Fe^{2+} or Cd^{2+} did not evoke further changes in the current response of the control oocyte. On the other hand, the addition of Fe^{2+} or Cd^{2+} at pH 5.5 caused a further inward current response in an scDMT-injected oocyte (Fig. 3b), suggesting that scDMT functions as a divalent metal transporter, as already reported for rat DMT (DCT1) [5]. Cd^{2+} transport activity of scDMT suggests its possible implication in the extraordinary cadmium accumulation under natural condition. Since the response to Fe^{2+} or Cd^{2+} was not evoked at pH 7.5 (data not shown), an increase in the extracellular H^+ concentration is required for metal transport of scDMT.

We next measured the divalent metal transport activity in the Ca^{2+}-containing medium. No inward current was detected in a non-injected control even in the presence of Fe^{2+} at pH 5.5 (Fig. 3c). In an scDMT-injected oocyte, on the other hand, shifting pH from 7.5 to 5.5 caused a significant inward current (Fig. 3d), which was possibly caused by Ca^{2+} (600 μM) contained in the medium. The addition of Fe^{2+} evoked a further inward current at pH 5.5, but an inward current was unexpectedly evoked immediately after the depletion of Fe^{2+} from the medium as shown by the closed arrow in Fig. 3d. This inward current was not observed in the Ca^{2+}-free medium after

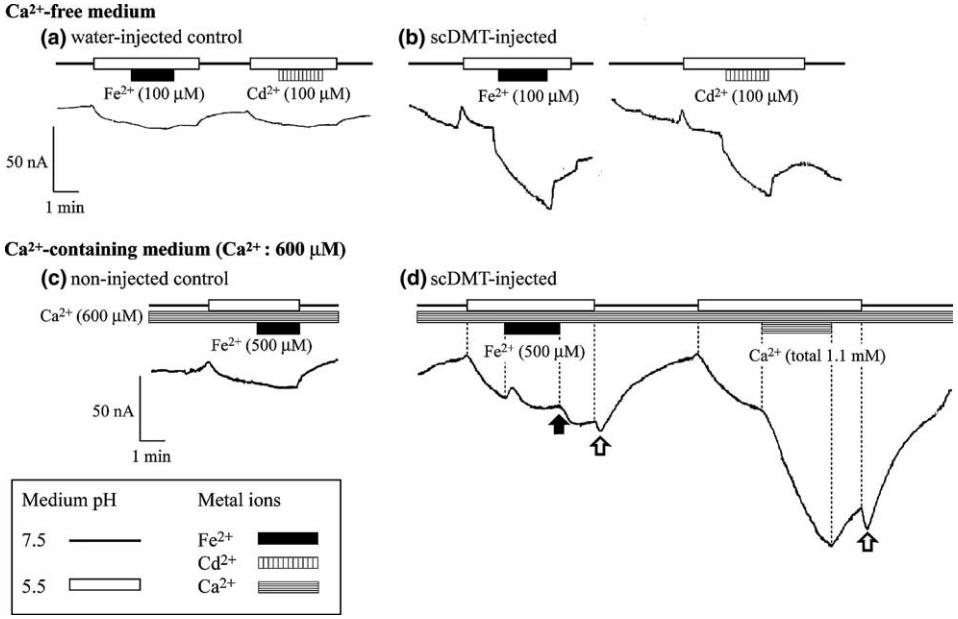

Fig. 3. Inward currents evoked by divalent metal ions in oocytes expressing scDMT. Current was continuously monitored in a water-injected control oocyte (a) and an oocyte expressing scDMT (b), both perfused with the Ca^{2+}-free medium, or a non-injected control oocyte (c) and an oocyte expressing scDMT (d), both perfused with the Ca^{2+}-containing medium. Oocytes were voltage-clamped at −50 mV and perfused with pH 7.5 medium and then perfused with pH 5.5 medium in the oocyte chamber for the times indicated by the open boxes, followed by the addition of divalent metal ions as indicated by the closed or shaded box. The closed arrow indicates the inward current evoked immediately after the depletion of Fe^{2+} from the medium. The open arrows indicate another inward currents evoked immediately after shifting pH from 5.5 to 7.5.

depletion of Fe^{2+} or Cd^{2+} (Fig. 3b), thereby ascribing to Ca^{2+} contained in the Ca^{2+}-containing medium. These facts suggest that scDMT functions as a Ca^{2+} transporter. To confirm this possibility, we examined the Ca^{2+} transport activity of scDMT. As a result, the addition of 500 μM Ca^{2+} (total 1.1 mM) evoked a significant inward current (Fig. 3d). It must be stressed that the depletion of added Ca^{2+} from the medium did not evoke the inward current that was observed immediately after Fe^{2+} depletion. On the other hand, another additional inward current shown by the open arrow in the figure was detected immediately after shifting pH from 5.5 to 7.5, which was also detected in case of Fe^{2+} transport experiment as well. Since these currents were not detected in the Ca^{2+}-free medium (Fig. 3b), they could be ascribed to Ca^{2+} in the Ca^{2+}-containing medium. We are now pursuing the molecular mechanism to evoke the additional currents after depletion of metal ions or shifting pH from 5.5 to 7.5 in the Ca^{2+}-containing medium.

Our first aim was to identify a transport molecule involved in the extraordinary accumulation of scallop. Although we have successfully cloned scDMT cDNA as a gene encoding a protein that transports Cd^{2+}, further studies are required to determine whether scDMT is responsible for the extraordinary cadmium accumulation. It seems probable that Cd^{2+} may be concomitantly transported by scDMT with essential divalent metal ions, detoxified by binding to metallothioneine and accumulated in the hepatopancreas [21]. Unexpectedly, scDMT was shown to have Ca^{2+} transport activity, although Ca^{2+} was reported to suppress the activity of rat DCT1 [5]. It is noteworthy that scDMT transported Ca^{2+} at the concentration of 1.1 mM that corresponds to the range found in seawater [22]. This finding suggests that scDMT physiologically functions as a Ca^{2+} transporter under natural condition. scDMT may function to concentrate Ca^{2+} from seawater for the process of biomineralization, because bivalves are supposed to require a large amount of Ca^{2+} for shell formation.

Acknowledgment: This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 16658086).

References

[1] Andrews, N.C., Fleming, M.D. and Gunshin, H. (1999) Iron transport across biologic membranes. Nutr. Rev. 57, 114–123.
[2] Vidal, S.M., Malo, D., Vogan, K., Skamene, E. and Gros, P. (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Cell 73, 469–485.
[3] Gruenheid, S., Cellier, M., Vidal, S. and Gros, P. (1995) Identification and characterization of a second mouse Nramp gene. Genomics 25, 514–525.
[4] Govoni, G. and Gros, P. (1998) Macrophage NRAMP1 and its role in resistance to microbial infections. Inflamm. Res. 47, 277–284.
[5] Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L. and Hediger, M.A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388, 482–488.
[6] Fleming, M.D., Romano, M.A., Su, M.A., Garrick, L.M., Garrick, M.D. and Andrews, N.C. (1998) Nramp2 is mutated in the anemic Belgrade (*b*) rat: evidence of a role for Nramp2 in endosomal iron transport. Proc. Natl. Acad. Sci. USA 95, 1148–1153.
[7] Fleming, M.D., Trenor, C.C., Su, M.A., Foernzler, D., Beier, D.R., Dietrich, W.F. and Andrews, N.C. (1997) Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat. Genet. 16, 383–386.
[8] Su, M.A., Trenor, C.C, Fleming, J.C., Fleming, M.D. and Andrews, N.C. (1998) The G185R mutation disrupts function of the iron transporter Nramp2. Blood 92, 2157–2163.
[9] Andrews, N.C. (1999) The iron transporter DMT1. Int. J. Biochem. Cell Biol. 31, 991–994.
[10] Lee, P.L., Gelbart, T., West, C., Halloran, C. and Beutler, E. (1998) The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. Blood Cells Mol. Disease 24, 199–215.
[11] Gunshin, H., Allerson, C.R., Polycarpou-Schwarz, M., Rofts, A., Rogers, J.T., Kishi, F., Hentze, M.W., Rouault, T.A., Andrews, N.C. and Hediger, M.A. (2001) Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett. 509, 309–316.
[12] Michibata, H., Uyama, T., Ueki, T. and Knamoria, K. (2002) Vanadocytes, cells hold the key to resolving the highly selective accumulation and reduction of vanadium in ascidians. Micros. Res. Tech. 56, 421–434.
[13] Evtushenko, Z.S., Belcheva, L.L. and Lukyanova, O.N. (1986) Cadmium accumulation in organs of the scallop *Mizuhopecten yessoensis* – II. Subcellular distribution of metals and metal-binding proteins. Comp. Biochem. Physiol. C 83, 377–383.
[14] Chelomin, V.P., Bobkova, E.A., Lukyanova, O.N. and Chekmasova, N.M. (1995) Cadmium-induced alterations in essential trace element homeostasis in the tissues of scallop *Mizuhopecten yessoensis*. Comp. Biochem. Physiol. C 110, 329–335.
[15] Leazer, T.M., Liu, Y. and Klaassen, C.D. (2002) Cadmium absorption and its relationship to divalent metal transporter-1 in the pregnant rat. Toxicol. Appl. Pharmacol. 185, 18–24.
[16] Park, J.D., Cherrington, N.J. and Klaassen, C.D. (2002) Intestinal absorption of cadmium is associated with divalent metal transporter 1 in rats. Toxicol. Sci. 68, 288–294.
[17] Sambrook, J. and Russel, D.W. (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[18] Kishi, F. and Tabuchi, M. (1997) Complete nucleotide sequence of human NRAMP2 cDNA. Mol. Immunol. 34, 839–842.
[19] Cellier, M., Prive, G.., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W. and Gros, P. (1995) Nramp defines a family of membrane proteins. Proc. Natl. Acad. Sci. USA 92, 10089–10093.
[20] Donovan, A., Brownlie, A., Dorschner, M.O., Zhou, Y., Pratt, S.J., Paw, B.H., Phillips, R.B., Thisse, C., Thisse, B. and Zon, L. (2002) The zebrafish mutant gene chardonnay (*cdy*) encodes divalent metal transporter 1 (DMT1). Blood 100, 4655–4659.
[21] Irato, P., Sanonito, G., Cassini, A., Piccinni, E. and Albergoni, V. (2003) Metal accumulation and binding protein induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the Lagoon of Venice. Arch. Environ. Contamin. Toxicol. 44, 476–484.
[22] Sverdrup, H.U., Johnson, M.W. and Fleming, R.H. (1961) The Oceans: Their Physics, Chemistry and General Biology pp. 165–227, Modern Asia Editions, Prince-Hall, New York.